SUPPLEMENT

Renal tubule Nedd4-2 deficiency stimulates Kir4.1/Kir5.1 and thiazide-*sensitive Na-Cl cotransporter (NCC) in distal convoluted tubule (DCT).*

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Methods

Genotyping

Tail DNA was PCR amplified. For *Kcnj10*, primers are forward 5'-TGATGTATCTCGATTGCTGC-3' and reverse 5'-CCCTACTCAATGCTCTTAAC-3', respectively (yielding a 550 bp product from the floxed *Kcnj10* gene and a 420 bp product from the wild-type allele. For *Nedd4l,* forward and reverse primers are 5'-TGAGCTCATTGCTTCACTTCC-3' and 5'-TTCATGCTCGAAGCCTTAGC-3', respectively (230 bp for floxed Nedd4l; 150 bP for WT). For Pax8rtTA, forward and reverse primers are 5' CCATGTCTAGACTGGACAAGA-3' and 5'-CAGAAAGTCTTGCCATGACT-3', respectively (a 220 bp product). For LC1-CRE, primers are forward 5'-TTTCCCGCAGAACCTGAAGATG-3' and reverse 5'-TCACCGGCATCAACGTTTTCTT-3', respectively (a 190 bp product).

Preparation of the DCT

Mice were sacrificed by cervical dislocation and the abdomen was opened to expose the left kidney. We perfused the left kidney with 2 ml L-15 medium (Life Technology) containing Type 2 collagenase (250 unit/ml) and then removed the collagenase-perfused kidney. The renal cortex was separated and further cut into small pieces for additional incubation in collagenase-containing L-15 media for 30-50 min at 37^{o}C. The tissue was then washed three times with fresh L-15 medium and transferred to an ice-cold chamber for dissection. The isolated DCT tubules were placed on a small cover glass coated with poly-lysine and the cover glass was placed on a chamber mounted on an inverted microscope.

qRT-PCR

The RNA extracted from total 50 μg renal cortex of each group was isolated with RNeasy Mini kit (Qiagen). The cDNA was generated with Maxima First Strand cDNA kits (Fermentas, ThermoFisher Scientific). Briefly, 4μl of 5X Reaction Mix, 2μl of Maxima enzyme and 1 μg RNA was mixed. The mixture was incubated for 25°C for 10 min followed by 50°C for 30 mins. The NCC primers (2.5 μl, 12.5 nM) were mixed with 2 μl cDNA (200 ng) and 12.5 μl 2× SYBR Green master. MxPro3000 (Stratagene) was used to carry out the experiments, and we used $2^{-\Delta\Delta CT}$ to analyze the comparative expression level of NCC. GAPDH was used as a control. We repeat with primers for NCC and Gapdh from OriGene (Rockville, MD). The sequence of NCC is TGATGGCTTCAAGGACGAGG (forward) and TTCCAATGGGCAGGGTAAGG (reversed). The sequence of Gapdh is CATCACTGCCACCCAGAAGACTG (forward); and ATGCCAGTGAGCTTCCCGTTCAG (reversed).

Antibodies

Antibodies for pNCC (Thr53) (Catalog #: p1311-53, 1:3000 dilution, Phospho Solution), NCC (AB3553, 1:2000 dilution, Millipore), ENaCα (Catalog # SPC-403, 1:1000 dilution, Stressmarq), Kir4.1(Cat # APC-035, 1:400 dilution, Alomone), Nedd4-2 (Catalog #4013,

1:1000 dilution, Cell Signaling), GAPDH (Catalog #2118, 1:1000 dilution, Cell Signaling) and β-actin (ab8226, 1:5000 dilution, Abcam).

Results

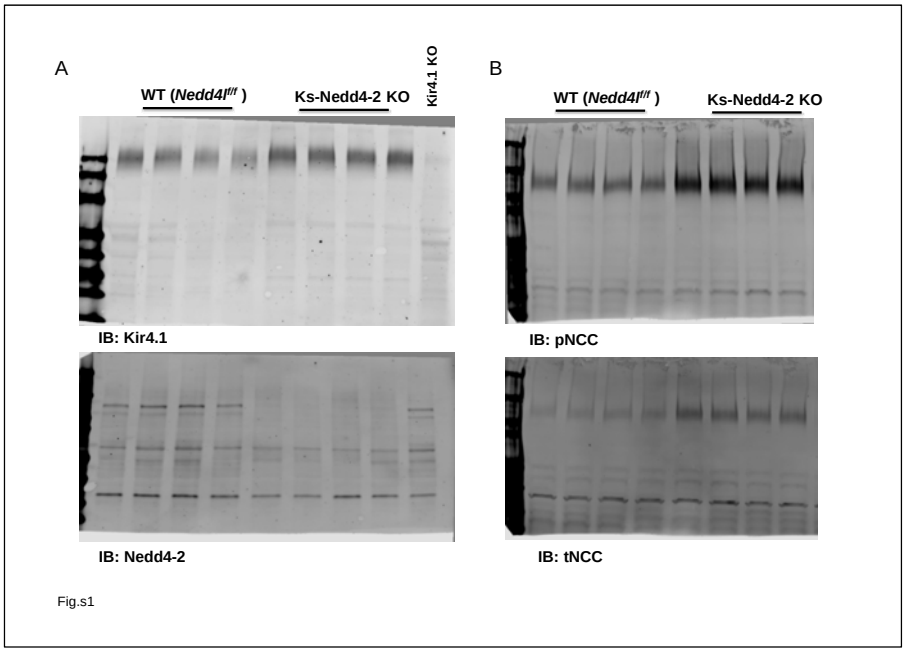


Fig.s1 **Deletion of Nedd4-2 increases the expression of Kir4.1 and NCC.** An immunoblot showing the expression of Kir4.1(A), Nedd4-2 (A), pNCC (B) and tNCC (B) in WT and Ks-Nedd4-2 KO mice. The sample from Ks-Kir4.1 KO mice serves as a negative control for Kir4.1.

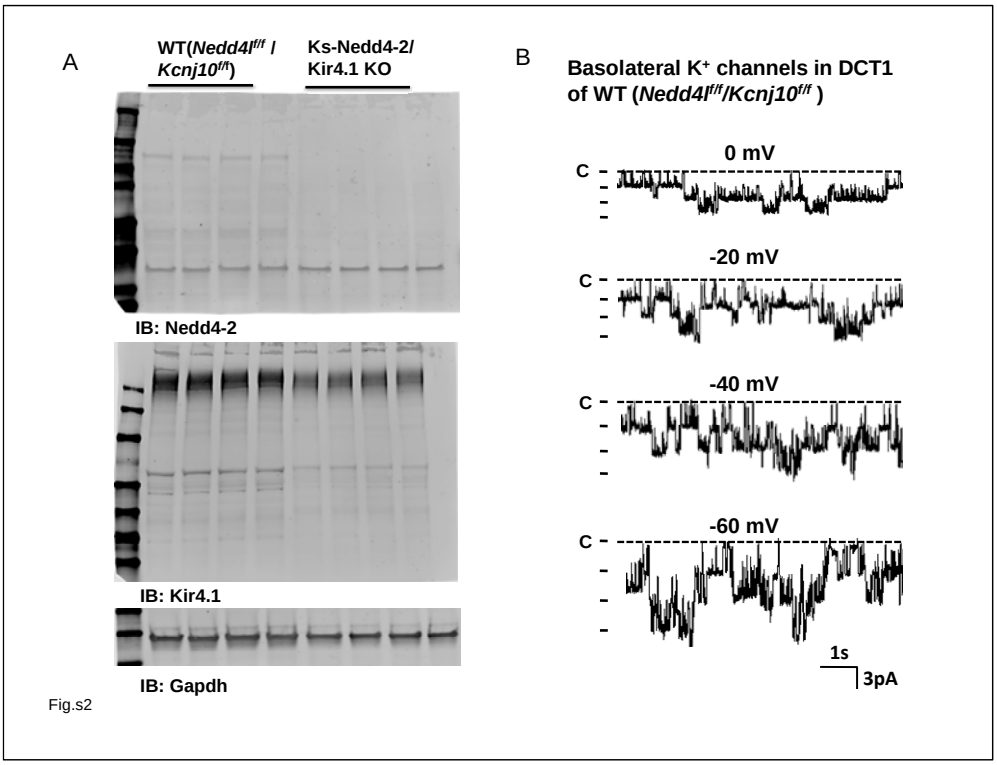


Fig. s2 **Basolateral 40 pS K^+ channel in the DCT of floxed *Nedd4l/Kcnaj10* (WT) mice.** (A) A western blot shows the expression of Nedd4-2 and Kir4.1 in WT and Ks-Nedd4-2/Kir4.1 KO mice. (B) A single channel recording shows the 40 pS K^+ channel activity in the basolateral membrane of the DCT from *Nedd4l*$^{flox/flox}$/ *Kcnj10*$^{flox/flox}$ (WT) mouse. The experiments were performed in a cell-attached patch with 140 mM Na^+/5 mM K^+ in the bath and 140 mM K^+ in the pipette. The channel closed level is indicated by "c" and holding potential was 0 mV.

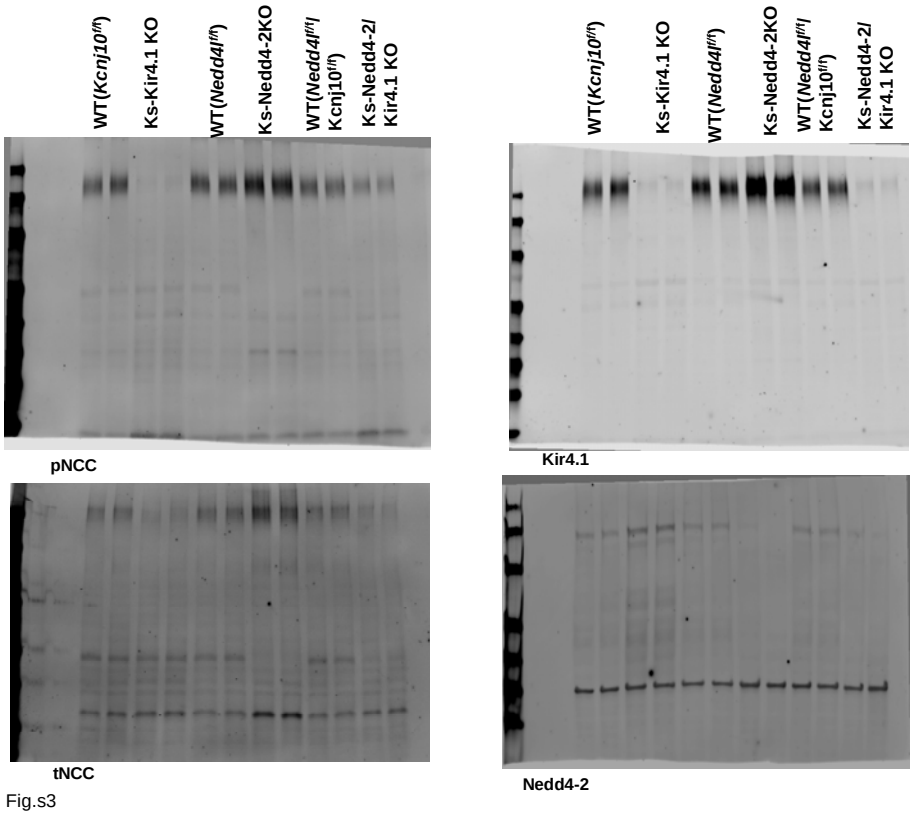


Fig.s3

Fig.s3 **Expression of pNCC and tNCC in Ks-Nedd4-2/Kir4.1 KO mice.** Full-size western blots show the expression of phosphorylated NCC (pNCC) , total NCC (tNCC), Kir4.1 and Nedd4-2 in WT(*Kcnj10*$^{flox/flox}$, *Nedd4l*$^{flox/flox}$ and *Nedd4l*$^{flox/flox}$/ *Kcnj10*$^{flox/flox}$), Ks-Kir4.1 KO, Ks-Nedd4-2 KO and Ks-Nedd4-2/Kir4.1 KO mice, respectively.

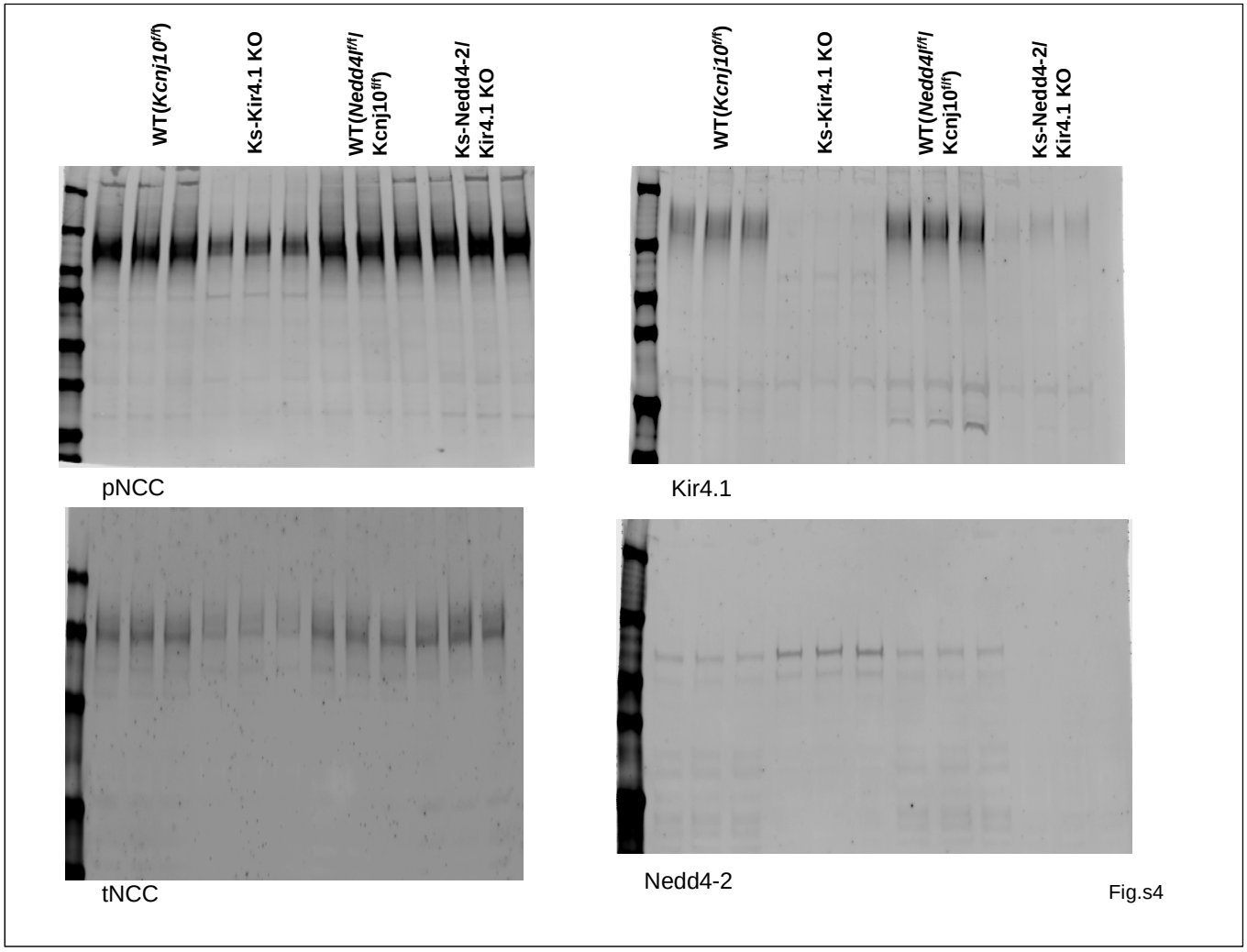


Fig. S4 **Expression of NCC in the mice at zero day after doxycycline treatment**. A immunoblot showing the expression of phosphorylated NCC (pNCC) , total NCC (tNCC), Kir4.1 and Nedd4-2 in WT (*Kcnj10*$^{flox/flox}$ and *Nedd4l*$^{flox/flox}$/ *Kcnj10*$^{flox/flox}$), Ks-Kir4.1 KO and Ks-Nedd4-2/Kir4.1 KO mice immediately after treatment of doxycycline (without 14 days transition period).

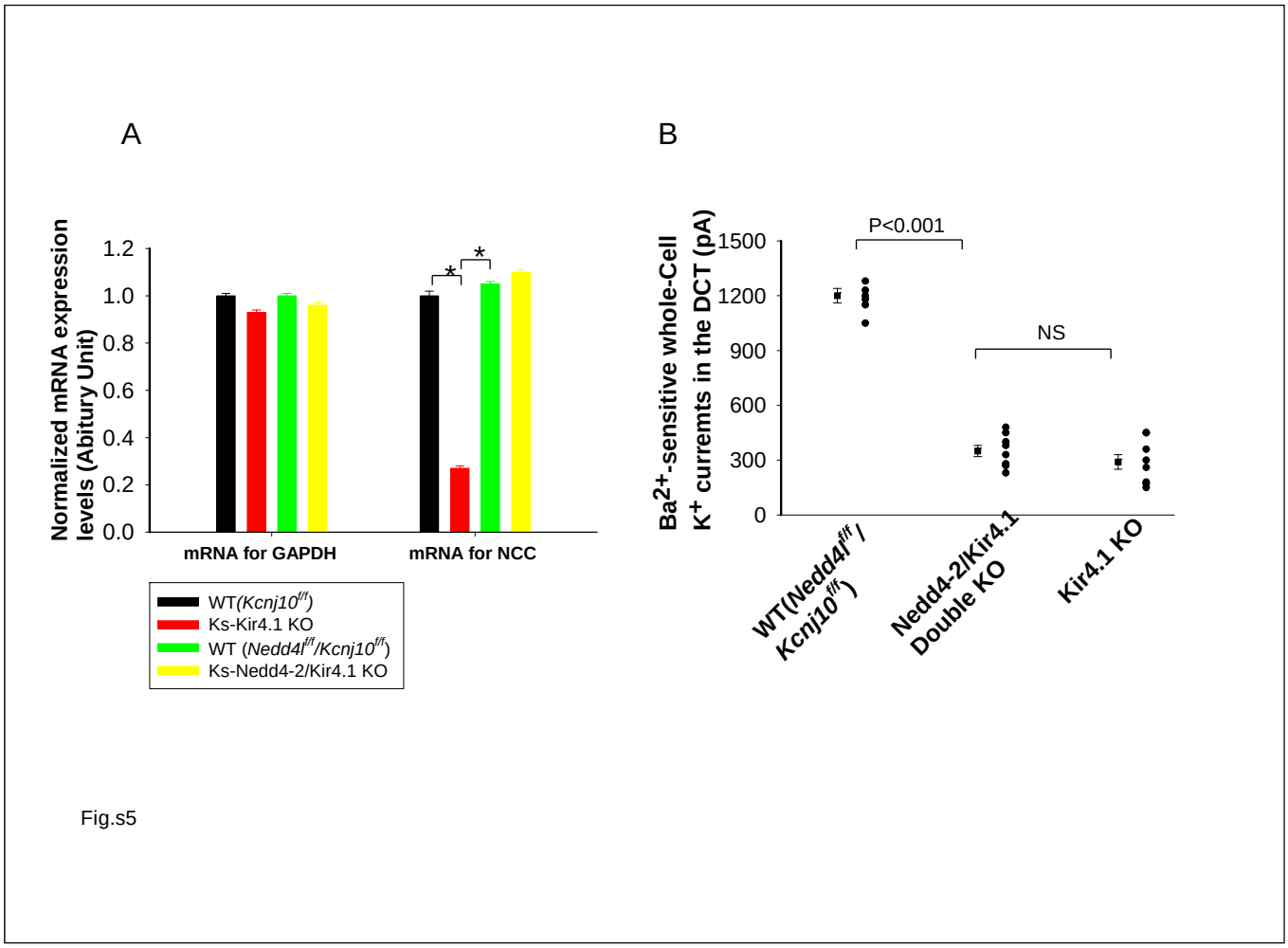
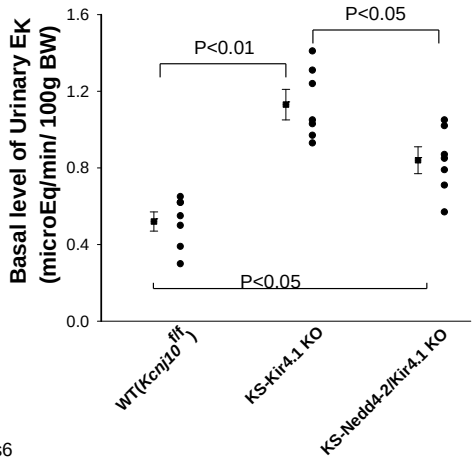


Fig.S5 **NCC mRNA expression and Kir4.1/Kir5.1 activity** in the mice at zero day after doxycycline treatment**.** A bar graph showing mRNA expression of GAPDH and NCC (A) in WT (*Kcnj10*$^{flox/flox}$ and *Nedd4l*$^{flox/flox}$/ *Kcnj10*$^{flox/flox}$). Ks-Kir4.1 KO and Ks-Nedd4-2/Kir4.1 KO mice (4 male mice for each group). (B) A scatter plot summarizes the results of experiments in which Ba^{2+}-sensitive whole-cell K^+ currents in the DCT were measured at -60 mV with step protocol in WT, Ks-Nedd4-2/Kir4.1 KO and Ks-Kir4.1 KO mice. The mice were used for experiments immediately after doxycycline treatment. Asterisk indicates significant difference determined by one-way ANOVA.

A **Plasma K^+ of WT and KO mice at 14th Day after doxycycline**

	WT(*Nedd4l*$^{f/f}$)	Ks-Nedd4-2 KO	WT (*Nedd4l*$^{f/f}$ */Kcnj10*$^{f/f}$)	Ks-Nedd4-2 /Kir4.1 KO	Ks-Kir4.1 KO
Plasma K^+(mM)	3.91±0.07 (n=5)	3.78±0.05 (n=6)	3.97±0.08 (n=6)	2.83±0.07* (n=6)	2.54±0.06# (n=5)
Plasma Na^+(mM)	147.4±1.8 (n=5)	145.6±0.6 (n=6)	147.5±0.6 (n=6)	147.8±0.6 (n=5)	147.4±1.0 (n=5)

B **E_K at Day zero after doxycycline**



C **Plasma K^+ at Day zero after doxycycline**

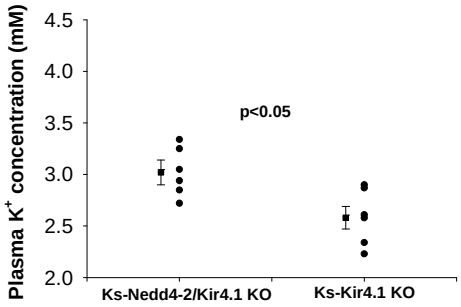


Fig.s6

Fig.S6 **Ks-Nedd4-2/Kir4.1 KO mice were hypokalemic**. (A) A table summarizes plasma K^+ concentration and plasma Na^+ concentrations in WT (*Nedd4l*$^{flox/flox}$ and *Nedd4l*$^{flox/flox}$ *Kcnj10*$^{flox/flox}$) Ks-Nedd4-2 KO, Ks-Nedd4-2/Kir4.1 KO and Ks-Kir4.1 KO mice, respectively. Asterisk indicates a significant difference (P<0.05) in comparison to WT control and # indicates a significant difference in comparison to all others. (B) A scatter plot shows the basal level of urinary K excretion (E_K) in WT (*Kcnj10*$^{f/f}$), Ks-Kir4.1 KO mice and Ks-Nedd4-2/Kir4.1 KO mice, respectively. The mice were used at day zero after treatment with vehicle or doxycycline for 14 day. (C) A scatter plot shows the plasma K^+ concentration in Ks-Kir4.1 KO and in Ks-Nedd4-2/Kir4.1 KO mice which were used immediately after doxycycline treatment.